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(54) Title: METHODS OF REDUCING APOLIPOPROTEIN E4-INDUCED INHIBITION OF NEURON REMODELING			
(57) Abstract <p>The present invention provides methods of reducing apolipoprotein E4 (apoE4) induced inhibition of neuron remodeling. The methods include preventing apoE4 from interacting effectively with neuronal low density lipoprotein receptor-related protein.</p>			

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**METHODS OF REDUCING APOLIPOPROTEIN E4-INDUCED INHIBITION  
OF NEURON REMODELING**

10 This invention was funded in part with funds from National Institutes of Health  
Program Project Grant HL41633. The Government may have certain rights to this  
invention.

Description

15 This is a continuation-in-part of United States patent application serial no.  
60/005,550, filed October 17, 1995, which is hereby incorporated in its entirety herein by  
reference.

Technical Field

20 This invention relates to the treatment of central and peripheral nervous system  
disorders relating to neuron remodeling. Specifically, the invention relates to the  
reduction of inhibition of neuron remodeling by interfering effectively with the  
interaction of apolipoprotein E4 (apoE4) with the neuronal low density lipoprotein  
receptor-related protein (LRP) or similar apoE binding receptor. In addition, the  
invention provides methods for reducing the apoE4 effects on neurons converting apoE4  
25 to an "apoE3-like" molecule with respect to receptor binding activity, cytoskeletal  
assembly/stability/activity and neurite extension or remodeling.

### Background

ApoE, a 34,000 molecular weight protein is the product of a single gene on chromosome 19 and exists in three major isoforms designated apoE2, apoE3 and apoE4 for review, see Mahley (in press) in: Molecular and Genetic Bases of Neurological Disease 2nd ed.; and Mahley (1988) Science 240:622-630. The different isoforms result from amino acid substitutions at amino acid residue positions 112 and 158. The common isoform, apoE3, has a cysteine residue at position 112 and an arginine residue at position 158. The apoE4 isoform differs from apoE3 only at position 112, which is an arginine residue. The apoE2 isoform, associated with type III hyperlipoproteinemia (Mahley (1988)), differs from apoE3 only at position 158, which is a cysteine residue. ApoE3 and apoE4 bind normally to the low density lipoprotein (LDL) receptor, whereas apoE2 does not.

ApoE contains two structural domains: an amino-terminal and a carboxy-terminal domain. Weisgraber (1994) Adv. Protein Chem. 45:249-302. Each domain is associated with a specific function. The amino terminal domain contains the lipoprotein receptor binding region and the carboxy-terminal domain contains the major lipid-binding elements. The two domains appear to interact with each other in an isoform-specific manner such that amino acid substitutions in one domain influence the function of the other domain, a phenomenon referred to as domain interaction. Domain interaction is responsible for the preference of apoE4 for very low density lipoproteins (VLDL) contrasted with the preference of apoE3 for high density lipoproteins (HDL). The specific amino acid residues in apoE4 that are involved in this interaction have been identified: arginine-61 in the amino-terminal domain and glutamic acid-255 in the carboxy-terminal domain. Dong et al. (1994) J. Biol. Chem. 269:22358-22365; and Dong and Weisgraber (1995) Circulation 92:I-427-I-428 (abstract).

By redistributing lipids among the cells of different organs, apoE plays a critical role in lipid metabolism. While apoE exerts this global transport mechanism in

chylomicron and VLDL metabolism, it also functions in the local transport of lipids among cells within a tissue. Cells with excess cholesterol and other lipids may release these substances to apoE-lipid complexes or to HDL containing apoE, which can transport the lipids to cells requiring them for proliferation or repair. The apoE on these lipoprotein particles mediates their interaction and uptake via the LDL receptor or the LRP.

ApoE plays a neurobiological role. ApoE mRNA is abundant in the brain, where it is synthesized and secreted primarily by astrocytes. Elshourbagy et al. (1985) Proc. Natl. Acad. Sci. USA 82:203-207; Boyles et al. (1985) J. Clin. Invest. 76:1501-1513; and Pitas et al. (1987) Biochem. Biophys. Acta 917:148-161. The brain is second only to the liver in the level of apoE mRNA expression. ApoE-containing lipoproteins are found in the cerebrospinal fluid and appear to play a major role in lipid transport in the central nervous system (CNS). Pitas et al. (1987) J. Biol. Chem. 262:14352-14360. In fact, the major cerebrospinal fluid lipoprotein is an apoE-containing HDL. ApoE plus a source of lipid promotes marked neurite extension in dorsal root ganglion cells in culture. Handelsmann et al. (1992) J. Lipid Res. 33:1677-1688. ApoE levels dramatically increase (about 250-fold) after peripheral nerve injury. Müller et al. (1985) Science 228:499-501; and Ignatius et al. (1986) Proc. Natl. Acad. Sci. USA 83:1125-1129. ApoE appears to participate both in the scavenging of lipids generated after axon degeneration and in the redistribution of these lipids to sprouting neurites for axon regeneration and later to Schwann cells for remyelination of the new axons. Boyles et al. (1989) J. Clin. Invest. 83:1015-1031; and Ignatius et al. (1987) Science 236:959-962.

Most recently, apoE has been implicated in Alzheimer's disease and cognitive performance. Saunders et al. (1993) Neurol. 43:1467-1472; Corder et al. (1993) Science 261:921-923; and Reed et al. (1994) Arch. Neurol. 51:1189-1192. ApoE4 is associated with the two characteristic neuropathologic lesions of Alzheimer's disease; extracellular neuritic plaques representing deposits of amyloid beta (A $\beta$ ) peptide and intracellular

neurofibrillary tangles representing filaments of hyperphosphorylated tau, a microtubule-associated protein. For review, see, McKhann et al. (1984) Neurol. 34:939-944; Selkoe (1991) Neuron 6:487-498; Crowther (1993) Curr. Opin. Struct. Biol. 3:202-206; Roses (1994) Curr. Neurol. 14:111-141; Weisgraber et al. (1994) Curr. Opin. Lipidol. 5:110-116; and Weisgraber et al. (1994) Curr. Opin. Struct. Biol. 4:507-515.

Alzheimer's disease is generally divided into three categories: early-onset familial disease (occurring before 60 years of age and linked to genes on chromosomes 21 and 14); late-onset familial disease; and sporadic late-onset disease. Both types of late-onset disease have recently been linked to chromosome 19 at the apoE locus. Other results suggest that apoE4 is directly linked to the severity of the disease in late-onset families. Roses (1994). Recently, cholesterol lowering drugs, the statins, have been suggested for use in treating Alzheimer's disease by lowering apoE4 levels. WO 95/06470.

The neurofibrillary tangles, which are paired helical filaments of hyperphosphorylated tau, accumulate in the cytoplasm of neurons. Tau is a microtubule-associated phosphoprotein which normally participates in microtubule assembly and stabilization; however, hyperphosphorylation impairs its ability to interact with microtubules. Increased binding of tau by apoE has been suggested as a treatment for Alzheimer's disease. WO 95/06456.

*In vitro* tau interacts with apoE3, but not with apoE4. Strittmatter et al. (1994) Exp. Neurol. 125:163-171. The interaction of apoE3 with tau may prevent its hyperphosphorylation, thus allowing it to function normally in stabilizing microtubular structure and function. In the presence of apoE4, tau could become hyperphosphorylated and thus inactive, which could promote the formation of neurofibrillary tangles.

ApoE4 has recently been associated with decreased learning ability and impaired memory. Helkala et al. (1995) Neurosci. Letts. 191:141-144. ApoE4 has been found to be a strong predictor of the outcome of patients designated as having memory impairment. Note that, apoE4 has been described as a risk factor, rather than a diagnostic.

Peterson et al. (1995) JAMA 273:1274-1278; and Feskens et al. (1994) BMJ 309:1202-1206.

5 ApoE interacts with both the LDL receptor and the LRP and undoubtedly with other apoE-binding receptors on neurons. The LRP has been found to be increased after brain injury or glial cell conversion to neoplasia. Lopes et al. (1994) FEBS Lett. 338:301-305. The LRP was previously identified as the-macroglobulin receptor. Strickland et al. (1991) J. Biol. Chem. 266:13364-13369; and Borth (1992) FASEB J. 6:3345-3353.

10 ApoE does not directly bind to the LRP but must first associate with cell surface heparan sulfate proteoglycans (HSPG). Mahley et al. (1991) Curr. Opin. Lipidol. 2:170-176; and Ji et al. (1994) J. Biol. Chem. 269:2764-2772. The LRP also binds a number of other ligands, including t-PA, $\alpha_2$ -macroglobulin-protease complex, thrombospondin-1, *Pseudomonas* exotoxin A, the receptor associated protein (RAP) and lactoferrin. The LRP ligand binding sites have been at least partially described. Orth et al. (1994) J. Biol.  
15 Chem. 269:21117-21122; Godyna et al. (1995) J. Cell. Biol. 129:1403-1410; Kounnas et al. (1992) J. Biol. Chem. 267:12420-12423; Willnow et al. (1994) J. Cell Sci. 107:719-726; Meilinger et al. (1995) FEBS Lett. 360:70-74; Warshawsky et al. (1993) J. Biol. Chem. 268:22046-22054; and Willnow et al. (1994) J. Biol. Chem. 269:15827-15832.

20 It has previously been shown that incubation of dorsal root ganglion neurons in culture with  $\beta$ -VLDL alters the neurite growth of these cells compared to that of cells grown in media alone. Handelsmann et al. (1992). In the presence of a source of lipid ( $\beta$ -VLDL or free cholesterol), neurite outgrowth is greatly enhanced, specifically due to extensive branching (with little or no increased neurite extension). When the  $\beta$ -VLDL  
25 was enriched with exogenous rabbit apoE (equivalent to human apoE3 with respect to the occurrence of a cysteine residue at position 112) enhanced neurite extension was seen. A lipid source appears to enhance membrane biosynthesis, whereas the addition of excess rabbit apoE with a lipid source results in long neuritic extensions and a trimming back of the branches. It has also been found that the inhibitory effect of apoE4 on neurite

outgrowth is associated with microtubule polymerization, whereas apoE3 supports microtubule formation. Nathan et al. (1995) J. Biol. Chem. 270:19791-19799.

5 Neural plasticity, maintenance of existing or formation of new synaptic connections, is critical for normal brain function, including memory. This process can be compromised by various forms of stress, including, but not limited to, age, deposition of plaques and neurofibrillary tangles in Alzheimer's disease and oxygen deprivation. Interference with neuron remodeling can lead to impaired brain function or  
10 neurodegeneration of which dementia and Alzheimer's disease are extreme examples. In the case of Alzheimer's disease alone, approximately 4 million individuals are affected in the United States. With the aging of the population, this number is projected to triple in the next twenty years. The present health care cost of Alzheimer's disease is estimated at \$90 billion per year in the United States alone. Delaying the average onset of this disease  
15 for even ten years would drastically reduce the financial burdens on society and the financial and emotional burdens of the families of these patients.

There are currently no effective therapies for arresting (and, more importantly, reversing) the impairment of central and peripheral nervous system function once an irreversible degenerative cascade begins. Likewise, there is no current therapy for  
20 restoration of normal, central and peripheral nervous system function when the induced stress has a less catastrophic or partially reversible effect compared to the dementias.

All references cited herein, both supra and infra, are hereby incorporated herein by reference.

#### Disclosure of the Invention

25 The present invention encompasses methods of reducing apoE4-induced inhibition of neuron remodeling. One method comprises administering to a patient in need thereof, an effective amount of a therapeutic agent (drug) which interferes with the interaction of apoE4 and neuronal apoE-binding receptors such as the LRP. As apoE4 interacts with the LRP by first associating with HSPG on the cell surface and then exerting its effect on the

LRP, the interaction can be reduced by interfering with either type of interaction both directly and indirectly.

5 In another method, apoE4 is "converted" to an "apoE3-like" molecule with respect to receptor binding activity, cytoskeletal assembly/stability/activity, and neurite extension or remodeling.

The invention also includes methods of identifying compounds that are effective in interfering with the apoE4 domain interaction. These methods are exemplified by the plasma distribution assay comprising the steps of adding a tracer dose of  $^{125}\text{I}$ -labeled apoE  
10 to plasma, separating the various plasma lipoprotein fractions by gel filtration and determining the distribution of  $^{125}\text{I}$ -label among lipoprotein classes. See, e.g. Dong et al. (1994) J. Biol. Chem. 269:22358-22365.

#### Brief Description of the Drawings

15 Figure 1 is a schematic representation of the human apoE cDNA constructs used to transfect the Neuro-2a cells. NSE promoter (N), exons of apoE have "E" underneath, the polylinker region has "P" underneath and apoE cDNA has "A" underneath.

Figure 2 is two photomicrographs of representative Neuro-2a cells stably transfected with apoE3 (A) or apoE4 (B) cDNA and grown for 96 hr in N2 medium  
20 containing  $\beta$ -VLDL (40  $\mu\text{g}$  of cholesterol/ml).

Figure 3 is a series of bar graphs depicting the effect of  $\beta$ -VLDL on the number of neurites per cell (A), neurite branching (B), and neurite extension (C) from control Neuro-2a cells and from cells stably transfected to express apoE3 or apoE4. In each case, the solid black bars represent the control, the striped bars represent apoE3 expressing cells  
25 and the solid white bars represent apoE4 expressing cells. In all cases the X-axis represents  $\beta$ -VLDL ( $\mu\text{g}$  cholesterol/ml).

Figure 4 is a graph depicting the effect of  $\beta$ -VLDL on the percentage of cells expressing neurites. The cells were incubated as described for the results presented in

Fig. 3. Four different fields in each dish were selected, and the percentage of cells displaying neurites was measured. Data are the means of three different experiments performed in duplicate ( $\pm$  S.E.M.). The percentages of cells expressing neurites in the absence of  $\beta$ -VLDL were: control cells,  $35 \pm 11$  (open squares); apoE3-expressing cells,  $32 \pm 9$  (closed circles); apoE4-expressing cells,  $25 \pm 13$  (closed squares).  $*p < 0.025$  versus control;  $**p < 0.005$  versus control.

Figure 5 is a bar graph depicting the effect of cerebrospinal fluid (CSF) lipoproteins on neurite extensions from Neuro-2a cells stably transfected to express apoE3 or apoE4. Cells were incubated with  $\beta$ -VLDL or bovine CSF lipoproteins ( $d < 1.21$  g/ml) under the conditions described for the results presented in Fig. 3. Each data point represents the measurement of 20-40 neurons. The data are reported as the mean  $\pm$  S.E.M. The calculation of the level of significance of the differences observed was performed as described for the results obtained in Fig 3. The solid black bars represent the control. The striped bars represent apoE3 expressing cells. The solid white bars represent apoE4 expressing cells.  $*p < 0.025$ ,  $**p < 0.01$ ,  $***p < 0.005$ .

Figure 6 depicts two photomicrographs of internalization of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI)-labeled  $\beta$ -VLDL by Neuro-2a cells stably transfected with apoE3 (A) or apoE4 (B) cDNA. Cells were grown for 24 hr in N2 medium. Then DiI-labeled  $\beta$ -VLDL ( $5 \mu\text{g}$  of protein/ml medium) were added, and the incubation was continued for 5 hr at  $37^\circ\text{C}$ .

#### Modes for Carrying Out the Invention

In neurons, the cytoskeleton functions in neurite extension and retraction. Therefore, the studies described herein and by others (Handelmann (1992); and Nathan et al. (1994) *Science* 264:850-852), have focused on the isoform-specific effects of apoE3 and apoE4 on neurite extension and branching. It has now been found that apoE modulates the intracellular cytoskeletal apparatus and alters neurite extension and

branching. Understanding how the various apoE isoforms alter the cytoskeleton may shed light on the process of neurofibrillary tangle formation and allow control of apoE-induced remodeling of synaptic connections later in life. Stimulation of neurite extension  
5 *in vivo* is thought to promote nerve regeneration or the formation of synaptic connections during neuronal remodeling in both the central and peripheral nervous system.

A comparison of the effects of human apoE3 versus human apoE4 showed pronounced differential isoform-specific effects on neurite outgrowth. Nathan et al.  
10 (1994). Compared to a control, human apoE3 plus  $\beta$ -VLDL resulted in an increase in neurite extension, while apoE4 plus  $\beta$ -VLDL resulted in a marked decrease in both neurite branching and extension. Results presented by Nathan et al. (1995) show that dorsal root ganglion neurons incubated with apoE4 plus  $\beta$ -VLDL displayed very short, stunted neurites. This was not a toxic effect of apoE4 since replacement of the apoE4-  
15 containing media with fresh apoE4-lacking media restored the ability of the neurons to produce neuritic extensions. Furthermore, it has now been found that the apoE3- and apoE4-specific effects were blocked by addition of an antibody against the receptor binding domain of apoE or by reductive methylation of critical lysine residues, indicating that this effect of apoE is receptor-mediated.

Neuro-2a cells from the central nervous system were used to compare the effects  
20 of apoE on the peripheral nervous system neurons described above with the effect on cortical neurons. Cells of both types respond similarly to apoE. When combined with a source of lipid, apoE3 stimulated neurite extension, whereas apoE4 inhibited neurite extension. Nathan et al. (1994) Soc. Neurosci. 20 (Part 2):1033 (Abstr.); and Nathan et  
25 al. (1995). Addition of free apoE3 or apoE4 without  $\beta$ -VLDL had no effect on neurite outgrowth. These results further suggest that the effect of apoE on neurons requires the lipoprotein receptor-mediated uptake of apoE or a combination of apoE and lipid. Free of lipid, apoE does not bind to either the LDL receptor or the LRP. In contrast, in another study, using a different neuronal cell line, Holtzman et al. demonstrated that apoE3 with

$\beta$ -VLDL stimulated nerve growth factor-induced neurite outgrowth, whereas apoE4 had no effect. Holtzman et al. (1995) Soc. Neurosci. 21 (abstr):1009, 400.10.

5 The studies described in Nathan et al. (1995) were performed by adding large quantities of apoE along with  $\beta$ -VLDL to the cells in culture. To determine whether lower levels of endogenously produced apoE would have an effect on neurite outgrowth from Neuro-2a cells, in the examples provided below, the neuronal cells were transfected with human apoE cDNA constructs encoding apoE3 or apoE4. Clones of the transfected  
10 cells secreting equal amounts of apoE3 or apoE4 (~50-60 ng of apoE/mg of cell protein/48 hours) were selected for comparison. The apoE3- and apoE4-secreting cells grown in serum-free control medium displayed a similar degree of limited neurite extension. However, when a source of lipid ( $\beta$ -VLDL) was added to the medium, the cells had a markedly different growth pattern. The apoE3-secreting cells showed greater  
15 neurite extension than did the apoE4-secreting cells. Thus, even very low levels of endogenously produced apoE along with a source of lipid revealed the differential effects of apoE3 versus apoE4. Lipid emulsions of various compositions, as well as cerebrospinal fluid lipoproteins can be substituted for the  $\beta$ -VLDL and appear to serve as a source of lipid for the cells or as a vehicle for transporting the apoE into a specific  
20 intracellular pathway. The examples presented herein show that the apoE effect on neurite outgrowth is mediated through the LRP, or a similar apoE-binding receptor, and that blocking or effectively preventing this interaction inhibits the apoE4 induced inhibition of neurite outgrowth.

Thus, the invention relates to methods of reducing the apoE4-induced inhibition of  
25 neuron remodeling by reducing the interaction of apoE4 and an apoE-binding receptor, e.g., the LRP.

Further, the invention relates to altering the function of apoE4 by changing the domain interaction to interfere with the inhibition of apoE4 in neuron remodeling. Any agent that blocks the interaction of arginine-61 with glutamic acid-255 in apoE4 is

suitable for use in this method. Blocking domain interaction in apoE4 converts apoE4 to an "apoE3-like" molecule, thereby blunting the undesirable effects of apoE4 on neurite extension. This may also have the effect of switching the apoE4 binding preference from VLDL to HDL.

Patients in need of such therapy are selected from those suffering from a wide range of disorders. For instance, patients particularly suitable for such therapy are those suffering from neurodegeneration or hypoxia. Neurodegeneration may result from a number of causes, including, but not limited to, Alzheimer's disease, trauma, viral infections, genetic enzyme deficiencies, age-related cognitive decline, and prion diseases. Viruses which may cause neurodegeneration include, but are not limited to, human immunodeficiency virus (HIV) and Epstein-Barr virus. Genetic enzyme deficiencies which may cause neurodegeneration include, but are not limited to, deficiency in  $\beta$ -N-acetylhexosaminidase which causes Tay-Sachs disease. Age-related cognitive decline is described, for instance, in Diagnostic and Statistical Manual of Mental Disorders, Fourth ed., Washington D.C. American Psychiatric Association (1994). Prion diseases include, but are not limited to, Kuru and Creutzfeldt-Jacob disease. Hypoxia is generally the result of stroke or is temporary and associated for instance with drowning, airway obstructions or carbon monoxide poisoning.

Neuron remodeling is also important in otherwise healthy patients. Therefore, the methods described herein may be suitable for use prophylactically in patients who are heterozygous or homozygous for apoE4 but do not show overt symptoms of Alzheimer's disease or other neurodegenerative disorders.

A variety of therapeutic agents are suitable for use in the present invention. As described in the examples below, heparinases, the RAP and lactoferrin all reduce or abolish apoE4-induced inhibition of neurite outgrowth. Also, suitable agents include those that bind specifically to apoE4 and prevent its domain interaction, i.e. small molecules or antibodies. Agents that disrupt the domain interaction can be selected from

a wide variety of molecules, including, but not limited to, small molecules, peptides and antibodies which are designed to bind to arginine-61 or glutamic acid-255 of apoE4. An assay for screening for agents that disrupt this domain interaction is described in Example 3, below. Essentially, any assay that determines whether apoE4 exhibits apoE3 activity is suitable for use herein.

Heparinases or other modifiers of HSPG are effective *in vitro* in ameliorating the effects of apoE4 on neuron remodeling. However, their pleiotropic effects render them unsuitable for human therapy. Nonetheless, effective therapeutic agents include HSPG analogs which bind to apoE4 to prevent its binding to neurons but do not exert substantial pleiotropic effects.

The RAP is a glycoprotein with an apparent molecular mass of 39-kD in humans. The RAP specifically associates with gp330 and the LRP, both of which are members of the LDL receptor gene family. Various RAPs and homologs thereof have been described and their functional domains have been mapped. For review see, Orlando et al. (1994) Proc. Natl. Acad. Sci. USA 91:3161-3165; and Warshawsky et al. (1995) Biochem. 34:3404-3415. The RAP, and portions thereof, are known to block the binding of the LRP to its ligand t-PA and  $\alpha_2$ -macroglobulin-protease complexes. Warshawsky et al. (1994) Ann. N.Y. Acad. Sci. pp. 514-517.

Lactoferrin has been shown to bind to the LRP, gp330, and HSPG. Willnow et al. (1994) J. Biol. Chem. 267:26172-26180; Mahley et al. (1994) Ann. N.Y. Acad. Sci. USA 737:39-52; and Ji et al. (1994a) Arterioscler. Thromb. 14:2025-2032. Lactoferrin appears to be cleared from the bloodstream by binding with LRP. Meilinger et al. (1995). Lactoferrin blocks binding of ligands to both the LRP and HSPG and blocks the HSPG-LRP pathway. This apparently occurs through the interaction of a region of concentrated positive charge on the lactoferrin with negatively-charged groups on the HSPG and negatively-charged amino acids in the ligand binding domain of the LRP.

As described below, antibodies specific for apoE block the apoE4 induced inhibition of neuron remodeling. Thus, antibodies to either apoE4 or the LRP can be used therapeutically. In addition, antibodies that inhibit apoE4 domain interaction can be used therapeutically. As described below, methods are known in the art to determine whether an antibody inhibits the neuron remodeling inhibitory effect of apoE4 whether by inhibiting binding to the LRP or by altering the function of apoE4 to become more apoE3-like. Preferably, the antibody is monoclonal. More preferably, the antibody is monoclonal and specific for the apoE4 isoform and not apoE3 or apoE2. The term "antibody" also includes functional portions and equivalents thereof. For instance, antibodies include any monospecific compound comprised of a sufficient portion of the light chain variable region to effect binding to the epitope to which the whole antibody has binding specificity. The fragments may include the variable region of at least one heavy or light chain immunoglobulin peptide, and include, but are not limited to, Fab fragments, Fab2 fragments, and Fv fragments. In addition, the monospecific domains of antibodies can be produced by recombinant engineering. Such recombinant molecules include, but are not limited to, fragments produced in bacteria, and murine antibodies in which the majority of the murine constant regions have been replaced with human antibody constant regions.

An effective amount of a therapeutic agent is one which, in *in vitro* assays, reduces apoE4 inhibition of neurite outgrowth by at least about 10%, preferably at least about 50% and most preferably, at least about 90%. The effect on neurite outgrowth can be measured, for instance, by the methods described herein.

The therapeutic agent prevents apoE4 from interacting effectively with neuronal LRP or other apoE-binding receptors. This prevention can be directed at either the HSPG and/or the LRP interactions or by modifying its function to be more apoE3-like and can directly or indirectly block binding or otherwise prevent the signal transduction induced by apoE4 binding. Thus, the therapeutic agents described herein are considered to be

effective if they prevent inhibition of neurite outgrowth by any of these routes. Thus, whole proteins, any functional portion thereof, analog or homologue is suitable for use provided it prevents effective interaction of apoE4 and HSPG or LRP, or other apoE-binding receptors. For instance, changes in the amino acid sequences of the RAP or lactoferrin and other known ligands of the LRP, or other apoE-binding receptors, that do not substantially affect their ability to effectively block the interaction of apoE4 and the LRP are within the scope of the invention. For instance, the invention encompasses changes in proteins that result in conservative substitutions of amino acid residues, one or a few amino acid deletions or additions, and substitution of amino acid residues by amino acid analogs which do not significantly affect its properties.

Amino acid residues which can be conservatively substituted for one another include but are not limited to: glycine/alanine; valine/isoleucine/leucine; asparagine/glutamine; aspartic acid/glutamic acid; serine/threonine; lysine/arginine; and phenylalanine/tyrosine. Any conservative amino acid substitution which does not significantly affect the properties of compositions is encompassed by the present invention.

It is within the skill of one in the art to determine whether a particular agent has therapeutic utility, for instance, by utilizing the methods described herein. It is also within the skill of one in the art to formulate suitable dosage formats for delivery of the therapeutic agents. When the site of delivery is the brain, the therapeutic agent must be capable of being delivered to the brain.

The blood-brain barrier limits the uptake of many therapeutic agents into the brain and spinal cord from the general circulation. Molecules which cross the blood-brain barrier use two main mechanisms: free diffusion; and facilitated transport. Because of the presence of the blood-brain barrier, attaining beneficial concentrations of a given therapeutic agent in the CNS may require the use of drug delivery strategies. Delivery of therapeutic agents to the CNS can be achieved by several methods.

One method relies on neurosurgical techniques. In the case of gravely ill patients such as accident victims or those suffering from various forms of dementia, surgical intervention is warranted despite its attendant risks. For instance, therapeutic agents can be delivered by direct physical introduction into the CNS, such as intraventricular or intrathecal injection of drugs. Intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Methods of introduction may also be provided by rechargeable or biodegradable devices. Another approach is the disruption of the blood-brain barrier by substances which increase the permeability of the blood-brain barrier. Examples include intra-arterial infusion of poorly diffusible agents such as mannitol, pharmaceuticals which increase cerebrovascular permeability such as etoposide, or vasoactive agents such as leukotrienes. Neuwelt and Rappoport (1984) Fed. Proc. 43:214-219; Baba et al. (1991) J. Cereb. Blood Flow Metab. 11:638-643; and Gennuso et al. (1993) Cancer Invest. 11:638-643.

Further, it may be desirable to administer the pharmaceutical agents locally to the area in need of treatment; this may be achieved by, for example, local infusion during surgery, by injection, by means of a catheter, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as silastic membranes, or fibers.

Another method involves pharmacological techniques such as modification or selection of a therapeutic agent to provide an analog which will cross the blood-brain barrier. Examples include increasing the hydrophobicity of the molecule, decreasing net charge or molecular weight of the molecule, or modifying the molecule, such as to resemble one normally transported across the blood-brain barrier. Levin (1980) J. Med. Chem. 23:682-684; Pardridge (1991) in: Peptide Drug Delivery to the Brain; and Kostis et al. (1994) J. Clin. Pharmacol. 34:989-996.

Encapsulation of the drug in a hydrophobic environment such as liposomes is also effective in delivering drugs to the CNS. For example WO 91/04014 describes a liposomal delivery system in which the drug is encapsulated within liposomes to which molecules have been added that are normally transported across the blood-brain barrier.

Another method of formulating the drug to pass through the blood-brain barrier is to encapsulate the drug in a cyclodextrin. Any suitable cyclodextrin which passes through the blood-brain barrier may be employed, including, but not limited to, J-cyclodextrin, K-cyclodextrin and derivatives thereof. See generally, U.S. Patent Nos. 5,017,566, 5,002,935 and 4,983,586. Such compositions may also include a glycerol derivative as described by U.S. Patent No. 5,153,179.

Yet another method takes advantage of physiological techniques such as conjugation of a therapeutic agent to a transportable agent to yield a new chimeric transportable therapeutic agent. For example, vasoactive intestinal peptide analog (VIPa) exerted its vasoactive effects only after conjugation to a monoclonal antibody (Mab) to the specific carrier molecule transferrin receptor, which facilitated the uptake of the VIPa-Mab conjugate through the blood-brain barrier. Pardridge (1991); and Bickel et al. (1993) Proc. Natl. Acad. Sci. USA 90:2618-2622. Several other specific transport systems have been identified, these include, but are not limited to, those for transferring insulin, or insulin-like growth factors I and II. Other suitable, non-specific carriers include, but are not limited to, pyridinium, fatty acids, inositol, cholesterol, and glucose derivatives. Certain prodrugs have been described whereby, upon entering the central nervous system, the drug is cleaved from the carrier to release the active drug. U.S. Patent No. 5,017,566.

The compounds in the instant invention may be used in conjunction with one or more of these methods to achieve the therapeutically desired result. The choices of method and dosage scheme thereof are within the skill of one in the art.

The invention also encompasses methods for detecting potential therapeutic agents that reduce the interaction of apoE4 and the LRP. The methods include in vitro ligand

blotting techniques. This can be performed following the separation of cell membrane proteins (which contain the LRP) or the LRP partially purified from membrane proteins for instance by nonreducing sodium dodecylsulfate-polyacrylamide gel electrophoresis and transfer to a nitrocellulose membrane. Methods of partial purification of the LRP are described, for instance, by Schneider et al. (1985) Met. Enzymol. 109:405-417. The membrane is then incubated with apoE and a lipoprotein (e.g.  $\beta$ -VLDL) which is labeled, for instance by biotinylation. Binding of the apoE- $\beta$ -VLDL complex to the membrane is then visualized using reagents that detect the label. Agents to be tested for their ability to block the interaction are added to the nitrocellulose together with apoE and  $\beta$ -VLDL to determine if the interaction is blocked.

The following examples are provided to illustrate, but not limit, the claimed invention.

#### EXAMPLE 1

##### Interaction of apoE with LRP and Effect on Neurite Outgrowth

The following materials and methods were used to obtain the results discussed below.

##### *Materials*

Dimyristoylphosphatidylcholine (DMPC), DME/F12 (1:1 mixture of Dulbecco's nutrient modified Eagle's medium and Ham's mixture F12), media supplements (progesterone, putrescine, selenite, and transferrin), sodium chlorate, heparinase, lactoferrin, triolein, and egg yolk phosphatidylcholine (type XI-E) were purchased from Sigma Chemical Co. (St. Louis, MO), fetal bovine serum (FBS), and insulin from Gibco (Grand Island, NY), suramin from Miles Inc. (FBA Pharmaceuticals, West Haven, CT), and DiI from Molecular Probes Inc. (Eugene, OR). Neuro-2a was purchased from American Type Culture Collection (Rockville, MD). Bovine CSF was obtained from Pel-Freez, Inc. (Fayetteville, AR).

*Preparation of Lipoproteins and Liposomes*

Rabbit  $\beta$ -VLDL ( $d < 1.006$  g/ml) were isolated from the plasma of New Zealand white rabbits fed a high-fat, high-cholesterol diet for four days according to the method described by Kowal (1989) Proc. Natl. Acad. Sci. USA 86:5810-5814. Rabbit VLDL ( $d < 1.006$  g/ml) were isolated by ultracentrifugation from fasting plasma obtained from rabbits fed a normal rabbit chow. The VLDL were washed once by ultracentrifugation at  $d = 1.006$  g/ml. Bovine CSF lipoproteins ( $d < 1.21$  g/ml) were isolated by ultracentrifugation according to the method described by Pitas et al. (1987) J. Biol. Chem. 262:14352-14360. They were washed once by recentrifugation through a solution of  $d = 1.21$  g/ml. Canine apoE HDL<sub>c</sub> ( $d = 1.006$ - $1.02$  g/ml) were isolated by ultracentrifugation and Pevikon electrophoresis from the plasma of foxhounds fed a semisynthetic diet containing hydrogenated coconut oil and cholesterol according to the method described by Mahley et al. (1977) Am. J. Pathol. 87:205-226. The  $\beta$ -VLDL were iodinated according to the method described by Bilheimer et al. (1972) Biochim. Biophys. Acta 260:212-221, and free iodine was removed by PD10 column chromatography.

The DMPC vesicles were prepared essentially according to the method described by Innerarity et al. (1979) J. Biol. Chem. 254:4186-4190. The DMPC alone (90 mg) or with the addition of cholesterol (10 mg) was dissolved in benzene and dried by lyophilization. The lyophilized material was then resuspended in 3 ml of 0.15 M NaCl, 10 mM Tris-Cl, and 1 mM EDTA (pH 7.6) and sonicated for 30 min at 37°C using a sonifier cell disrupter (Branson 450, Danbury, CT) equipped with a microtip and full setting at 7 (50 watts). Innerarity (1979). The material was centrifuged for 10 min at 2,000 rpm (37°C), and the supernatant was used for addition to cells. The lipid emulsion A was prepared according to the methods described Pittman et al. (1987) J. Biol. Chem. 262:2435-2442; and Spooner et al. (1988) J. Biol. Chem. 263:1444-1453. Briefly, the lipids were mixed together in the following ratio: 100 mg of triolein and 25 mg of egg yolk phosphatidylcholine and then dried under a stream of nitrogen. The pellet was then

resuspended in 5 ml of 10 mM Tris-Cl, 0.1 M KCl, and 1 mM EDTA (pH 8.0) buffer and sonicated according to the method described by Spooner et al. (1988). The material was then centrifuged for 10 min at 2,000 rpm. The composition of the final emulsion was 2.7:1 for triolein:phosphatidylcholine (wt:wt). The size and morphology of the emulsion particles were determined by negative staining electron microscopy.

#### *Preparation of Expression Vectors*

The expression vectors were assembled in the pBSSK plasmid (Stratagene, La Jolla, CA). The constructs contained the rat neuron-specific enolase (NSE) promoter (kindly provided by Dr. J. G. Sutcliffe, Scripps Clinic and Research Foundation, La Jolla, CA), which has been previously used to direct neuron-specific expression of the human amyloid precursor protein and  $\beta$ -galactosidase in transgenic mice. Quon et al. (1991) *Nature* 352:239-241; and Forss-Petter (1990) *Neuron* 5:187-197. In addition, the construct contained the first exon (noncoding), the first intron, and the first six bases of the second exon (prior to the initiation methionine) of the human apoE gene, followed by the apoE cDNA.

The apoE4 construct was identical except that it also contained the third intron (Fig. 1). The noncoding region of the fourth exon was downstream from the cDNA, followed by 112 bp of the 3'-flanking sequence of the human apoE gene that contains the polyadenylation signal. The apoE constructs for insertion in these expression vectors were kindly provided by Drs. S. Lauer and J. Taylor of the J. David Gladstone Institutes. The orientation of the cDNAs was confirmed by sequencing, using an Applied Biosystems automated sequencer. The final constructs were referred to as NSE-E3 (for apoE3 cDNA) and NSE-E4 (for apoE4 cDNA) (Fig. 1). Plasmid DNA was purified by two rounds of cesium chloride gradient ultracentrifugation according to the method described by Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. To test the constructs, Chinese hamster ovary cells and human embryonic kidney 293 cells were transiently

transfected (lipofectin-mediated), and the concentration of apoE in the medium was measured as described below. Similar levels of expression of apoE3 and apoE4 were achieved.

#### *Production of Stably Transfected Neuro-2a Cell Lines*

Cells at 20-30% confluence were cotransfected with pSV2neo and either NSE-E3 or NSE-E4 using a calcium phosphate precipitation protocol essentially as described by Chen et al. (1988) BioTechniques 6:632-638. Control cells were transfected with pSV2neo alone, following the same protocol. Stably transfected cells were selected by growth in DME/F12 media containing 10% FBS and 400 µg/ml of G418 (Geneticin, Gibco). Individual G418-resistant colonies were selected and expanded. Secretion of human apoE3 or apoE4 by the transfected cells was verified by Western blotting of the conditioned media.

#### *ApoE Quantitation*

Intracellular, cell-surface-bound, and secreted apoE were quantitated in cells maintained for 96 hr in N2 medium, a serum- and lipid-free medium (DME/F12 containing growth supplements as described in Bottenstein et al. (1980) Exp. Cell Res. 129:361-366), with or without added β-VLDL (40 µg cholesterol per ml). The medium was changed once at 48 hr. The secreted apoE reported is that present in the medium following the second 48 hr incubation. The media were collected and, after the addition of protease inhibitors, centrifuged to eliminate suspended cells. The cell monolayers were washed with PBS and incubated for 1 hr at 4°C with 2 ml of DMEM/F12 containing 25 mM Hepes and 10 mM suramin, a polyanion that is able to release apoE bound to the cell surface. Ji et al. (1994). The apoE was precipitated from the medium and the suramin extract by addition of 50 µg/ml of fumed silica (Sigma, St. Louis, MO) and centrifugation at 13,000 x g for 10 min.

Each pellet was washed three times with sterile water and dissolved in gel-loading buffer. Cellular apoE was extracted from the cells, following suramin removal of surface-

bound apoE, using STEN buffer (50 mM Tris-Cl, pH 7.6, containing 150 mM NaCl, 2 mM EDTA, 1% NP-40, 20 mM PMSF, and 5 µg/ml leupeptin). Samples were electrophoresed on 5-20% polyacrylamide gradient gels containing sodium dodecyl sulfate, according to the method described by Ji et al. (1994) J. Biol. Chem. 269:13429-13436. The proteins were transferred to nitrocellulose paper by blotting and treated with an anti-human apoE polyclonal antiserum (1:1,000 dilution) raised in rabbit (generously provided by Dr. K. H. Weisgraber, Gladstone Institutes). The nitrocellulose immunoblot was then incubated with donkey anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:5,000 dilution) (Amersham, Arlington Heights, IL). After washing to remove unbound antibody, the immunocomplex was detected using an ECL kit (Amersham), according to the manufacturer's instructions. Quantitation of the level of apoE bound, internalized, and secreted by the cells was accomplished by densitometric scanning (Ambis Scanner, San Diego, CA) and based on a standard curve of purified human plasma apoE3 and apoE4.

#### *Neurite Outgrowth*

Cells were grown in DME/F12 containing 10% FBS and G418 (400 µg/ml). On the day the experiment was initiated, the cells were subcultured into 35 mm plates in DME/F12 with 10% FBS. The cells were allowed to adhere to the plastic plates for 2 hr at 37°C, and then the culture medium was changed to N2 medium with or without increasing concentrations of lipoproteins. After 48 hr at 37°C, the media were replaced with the same medium (with or without lipoproteins), and the incubation was continued for an additional 48 hr. (The CSF lipoproteins were dialyzed against N2 medium prior to addition to the cells.) The cells then were washed with DME/F12 containing 0.2% BSA, nonspecifically stained for 1 hr at 37°C with Dil added in DMSO according to the method described by Nathan et al. (1994) Science 264:850-852, and fixed with 2.5% glutaraldehyde in PBS (v/v). Neurons were imaged in fluorescence mode with a confocal laser scanning system (MRC-600, BioRad, Hercules, CA), and the images were digitized

with an Image-1/AT image analysis system (Universal Images, West Chester, PA). The neuronal images were coded before characterization, and the following variables were measured: 1) number of neurites (defined as cell surface projections at least one-half the cell diameter) on each neuron; 2) neurite branching (the number of branch points on each neurite); and 3) neurite extension (the length of the longest neurite, measured from the cell body). Typically, in each experiment the neurites of 20 to 40 neurons per plate were measured and the results preserved as the mean  $\pm$  S.E.M.

In studies on the effect of the inhibitors of lipoprotein binding to the LRP, cells were incubated for 1 hr at 37°C in N2 medium containing the indicated concentrations of either lactoferrin, chlorate, or heparinase or with the receptor-associated protein (RAP). Then the  $\beta$ -VLDL were added, and the incubation was continued for a total of 96 hr. The reagents, except for  $\beta$ -VLDL, were re-added every 24 hr. The media and  $\beta$ -VLDL were replaced after 48 hr.

#### *Cell Association and Degradation of $^{125}$ I- $\beta$ -VLDL*

The cells were grown for 24 hr in 35 mm dishes in N2 medium alone. Then  $^{125}$ I- $\beta$ -VLDL (3  $\mu$ g of protein per ml of medium) were added, and the incubation was continued for 16 hr at 37°C. The medium was analyzed for TCA-soluble lipoprotein degradation products according to the method described by Goldstein et al. (1983) Met. Enzymol. 98:241-260. The cells were placed on ice, washed with PBS containing 0.2% BSA, and dissolved in 0.1 N NaOH. Lipoprotein cell association was determined by measuring cellular radioactivity using a gamma counter (Beckman Gamma 8000, Beckman Instruments, Fullerton, CA) and according to the method described by Goldstein et al. (1983).

#### *Cell Association of DiI-labeled $\beta$ -VLDL*

The cells were grown for 24 hr in 35 mm dishes in N2 medium. Then DiI-labeled  $\beta$ -VLDL (4  $\mu$ g of protein per ml of medium), was prepared according to the methods described by Pitas et al. (1983) Arteriosclerosis 3:2-12; and Pitas et al. (1981)

Arteriosclerosis 1:177-185, were added, and the incubation was continued for 5 hr at 37°C. The cells were then washed with PBS and fixed with 4% paraformaldehyde in PBS (v/v). Uptake of DiI-labeled  $\beta$ -VLDL was visualized by fluorescence microscopy. To quantitate the amount of DiI-labeled lipoprotein in the cells at the end of the incubation, the cells were scraped, using two 0.5 ml aliquots of PBS, and lyophilized. The DiI was extracted from the dried cell pellet with methanol and analyzed using a spectrofluorometer (excitation 520 nm, emission 570 nm). Pitas et al. (1983). Standards of DiI in methanol were used for quantitation.

#### *Association of ApoE with Lipid Particles*

ApoE3 and apoE4 were iodinated using Bolton-Hunter reagent (DuPont NEN, Boston, MA) according to the method described by Innerarity et al. (1983) J. Biol. Chem. 258:12341-12347, and then incubated with the lipid particles for 1 hr at 37°C. The samples were then fractionated by chromatography on a Superose 6 column (10/50 HR, Pharmacia Fine Chemicals, Uppsala, Sweden) and eluted with 1 mM EDTA in PBS at a constant flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected and analyzed for cholesterol and triglyceride, and the  $^{125}\text{I}$ -apoE content was measured in a Beckman 8000 counter (Beckman Instruments) and according to the method described by Dong et al. (1994) J. Biol. Chem. 269:22358-22365.

#### *Statistical Analysis*

Data were analyzed using a paired *t*-test.

## **RESULTS**

The levels of apoE secreted into the medium, bound to the cell surface, and accumulated intracellularly by the stably transfected Neuro-2a cells expressing human apoE3 or apoE4 were assessed by Western blot analysis and quantitated by densitometry. The results obtained are presented in Table. 1.

Table 1

*ApoE3 or apoE4 secreted, releasable by suramin, or present inside cells stably transfected with apoE3 or apoE4 cDNA*

5	Cells	Secreted	Releasable	Intracellular
	ApoE3-expressing		ng of apoE/mg of cell protein	
	Clone #1	54	6.2	140
	+ $\beta$ -VLDL	56	7.2	119
10	Clone #3	44	4.9	259
	+ $\beta$ -VLDL	45	4.3	251
	ApoE4-expressing			
	Clone #4	60	6.7	215
	+ $\beta$ -VLDL	63	5.3	231
	Clone #5	69	8.0	135
	+ $\beta$ -VLDL	62	6.5	128
15	Clone #6	89	5.2	111
	+ $\beta$ -VLDL	87	5.6	105

To obtain the results depicted in Table 1, transfected cells were incubated for 96 hr in medium with or without  $\beta$ -VLDL (40  $\mu$ g cholesterol/ml). The medium was changed at 48 hr. ApoE secreted in the last 48 hr, intracellular, and suramin-releasable (surface-bound) apoE were quantitated at the end of the 96 hr of incubation as described in Nathan et al. (1995). The data are the mean of two separate determinations. The duplicates did not differ by more than 12%.

The results depicted in Table 1 indicate that the cells secreted 44-54 ng of apoE3 and 60-89 ng of apoE4 per mg of cell protein in 48 hr. The apoE3- and apoE4-secreting cells had similar amounts of apoE bound to the cell surface (releasable by suramin treatment), ranging from 4.9 to 8.0 ng of apoE per mg of cell protein. The intracellular content of apoE in the two apoE3-expressing cell lines was 140 and 259 ng of apoE per

mg of cell protein. Similar amounts of intracellular apoE (111-215 ng/mg) were seen in the apoE4-expressing cell lines. The addition of  $\beta$ -VLDL to the cells did not have a significant effect on the amount of apoE secreted, surface-bound, or present within the apoE3- or apoE4-secreting cells (Table 1).

In initial experiments, two Neuro-2a cell lines that secreted similar amounts of apoE3 (clone 1, 54 ng/mg of cell protein) and apoE4 (clone 4, 60 ng/mg of cell protein) (Table 1) were used to examine neurite growth. When these cells were grown in N2 medium in the absence of  $\beta$ -VLDL, there were no apparent differences in neurite outgrowth between the apoE3- and apoE4-secreting cells. However, incubation of the cells in N2 medium containing  $\beta$ -VLDL resulted in a markedly different pattern in the neurite outgrowth from these cells. ApoE3-secreting cells incubated with  $\beta$ -VLDL developed long neurites (Fig. 2A), whereas in apoE4-secreting cells neurite outgrowth was suppressed (Fig. 2B).

Differences in neurite outgrowth in the absence and presence of increasing concentrations of  $\beta$ -VLDL were quantitated by measuring the number of neurites per cell, neurite branching, and neurite extension (Figs. 3A, B, and C, respectively). The values for the non-apoE transfected control cells incubated for 96 hr in N2 medium in the absence of  $\beta$ -VLDL are set at 100%. The expression of either apoE3 or apoE4 by the transfected Neuro-2a cells did not influence neurite number, branching, or extension when the cells were grown in N2 medium in the absence of added lipoprotein (Figs. 3A, B, and C). To obtain the results depicted in Fig. 3, cells (clone #1 for apoE3-expressing and clone #4 for apoE4 expressing) were incubated for 96 hr in N2 medium alone or in medium containing increasing concentrations of  $\beta$ -VLDL. The media were changed at 48 hr. The cells were stained with DiI and fixed, and the indicated parameters were measured. Each data point was obtained by the measurement of 20-50 cells expressing neurites in four separate experiments. The data are presented as the percentage of the value obtained with control cells with N2 medium alone. The data are the mean  $\pm$  the

S.E.M. As depicted in Fig. 3, the average values obtained with control cells incubated with N2 medium alone were: A: neurites per cell = 3; B: branch points per neurite = 2; C: average neurite length = 155  $\mu$ m.

For calculation of the level of significance for the effect of added  $\beta$ -VLDL, the results in the presence of  $\beta$ -VLDL are, compared to the data obtained with the same cells in the absence of  $\beta$ -VLDL (i.e., grown in N2 medium alone). \* $p$  < 0.025; \*\* $p$  < 0.010; \*\*\* $p$  < 0.005.

However, as shown in Fig. 3A, the addition of  $\beta$ -VLDL resulted in an increase in the number of neurons in the control cells and in the cells secreting apoE3 (significantly increased at 40  $\mu$ g of  $\beta$ -VLDL cholesterol/ml compared with apoE3-secreting cells in N2 medium). On the other hand, in the presence of high concentrations of  $\beta$ -VLDL, the Neuro-2a cells secreting apoE4 showed a significant reduction in the number of neurites per cell as compared with the apoE4-secreting cells in the N2 medium.

As previously described for DRG cells (Handelmann et al. (1992) J. Lipids Res. 33:1677-1688; and Nathan et al. (1994)), the addition of  $\beta$ -VLDL alone resulted in increased branching of neurites. As shown in Fig. 3B, addition of  $\beta$ -VLDL to the non-apoE-transfected cells resulted in a significant increase in neurite branching. In addition, at the highest concentration of  $\beta$ -VLDL cholesterol, the apoE3-secreting cells displayed enhanced branching by comparison with the apoE3-secreting cells grown in N2 medium alone. In contrast, the apoE4-secreting cells tended to show decreased branching when incubated with  $\beta$ -VLDL; however, this decrease did not reach statistical significance.

Neurite extension was increased in the Neuro-2a cells secreting apoE3 when they were incubated with the highest concentrations of  $\beta$ -VLDL. In contrast, in the apoE4-secreting cells neurite extension was very significantly suppressed even at the lowest concentration of  $\beta$ -VLDL used (Fig. 3C).

The results described in Fig. 3 were based on a comparison of cells having neuritic outgrowths and did not take into account those Neuro-2a cells without neuritic extensions.

Approximately 25-30% of the Neuro-2a cells in N2 medium possessed neurite extensions (defined as a cell-surface projection of at least one-half the cell diameter). However, as shown in Fig. 4, it was apparent that in the presence of  $\beta$ -VLDL, the number of apoE3-secreting cells developing neurites increased markedly to 60-70% of the total. On the other hand, the number of apoE4-secreting cells developing neuritic extensions was significantly reduced, compared with the control or apoE3-secreting cells. Thus, the apoE3-secreting cells incubated with  $\beta$ -VLDL not only had longer neuritic extensions but also showed an increase in the number of cells with neurites. The apoE4-secreting cells grown in the presence of  $\beta$ -VLDL showed fewer neurites, and those that were produced were much shorter.

To ensure that the differential effect of  $\beta$ -VLDL on neurite outgrowth in the apoE3- and apoE4-secreting cells was not due to clonal variation or to differences in the secretion or intracellular content of apoE in the various cell lines, additional experiments were performed with the other stably transfected cell lines secreting apoE3 or apoE4. Incubation of these cells with  $\beta$ -VLDL also resulted in differential effects of apoE3 and apoE4 on neurite outgrowth. The results obtained are presented in Table 2.

Table 2

*Effect of  $\beta$ -VLDL (40  $\mu$ g cholesterol/ml medium) on the number of neurites per cell, neurite branching, and neurite extension from cells stably transfected with apoE3 or apoE4*

Cell type	Number of Neurites (% of values obtained with control cells in N2 medium alone)	Branching	Extension
ApoE3-expressing			
Clone #1	165 $\pm$ 30	186 $\pm$ 39	186 $\pm$ 13
Clone #2	150 $\pm$ 25	180 $\pm$ 15	190 $\pm$ 23
Clone #3	170 $\pm$ 39	175 $\pm$ 20	180 $\pm$ 25
ApoE4-expressing			
Clone #4	43 $\pm$ 25	65 $\pm$ 26	41 $\pm$ 9
Clone #5	49 $\pm$ 15	70 $\pm$ 31	50 $\pm$ 15
Clone #6	53 $\pm$ 19	60 $\pm$ 25	45 $\pm$ 19

In Table 2, the level of secretion of apoE by clones #1, #3, #4, #5, and #6 is as described for Table 1. Clone #2 secreted 36 ng of apoE3/mg of cell protein/48 hr.

5 Surface-bound and internalized apoE was not quantitated for clone #2. The conditions for incubation with  $\beta$ -VLDL are as described for Fig. 3. Each data point was obtained by the measurement of 25-40 cells. The data are the mean  $\pm$  S.E.M.

10 As summarized in Table 2, in the presence of  $\beta$ -VLDL, all of the apoE4-secreting cells showed a significant reduction in the number of neurites expressed, branching, and neurite extension, whereas the apoE3-secreting cells displayed an increased number of neurites, increased branching, and increased extension as compared to cells grown in N2 medium lacking a source of lipoprotein.

15 To determine whether apoE4 blocks neurite extension in the presence of  $\beta$ -VLDL or whether it induces neurite retraction, the cells were incubated for 48 hr in N2 medium alone to stimulate neurite outgrowth. The medium was changed, and the cells incubated for an additional 48 or 96 hr in media with  $\beta$ -VLDL (40  $\mu$ g of cholesterol per ml). The addition of  $\beta$ -VLDL did not decrease the extension of neurites of apoE4-expressing cells compared with cells incubated in N2 medium alone. Therefore, apoE4 in the presence of  $\beta$ -VLDL, inhibits neurite extension directly and does not cause a retraction of neurites that have already extended.

20 Other lipoproteins were used to determine if any lipid vehicle carrying apoE would substitute for  $\beta$ -VLDL. Incubation of the apoE3- or apoE4-expressing cells with rabbit VLDL, a lipoprotein rich in triglyceride, resulted in similar effects on neurite extension as obtained with  $\beta$ -VLDL. The results are presented in Table 3.

Table 3

*Effect of  $\beta$ -VLDL, VLDL or lipid emulsions on neurite extension from cells stably transfected with apoE3 or apoE4 cDNA*

Treatment	Lipid composition (wt/wt/wt)	Mean Size (nm $\pm$ S.D.)	Control	ApoE3-expressing	apoE4-expressing
			% of value obtained with control cells in N2 medium alone		
N2 alone	,	,	100 $\pm$ 10	110 $\pm$ 15	115 $\pm$ 11
$\beta$ -VLDL	CHOL: $\mu$ g:PL (5.6:0.4:1)	43.7 $\pm$ 25.6	120 $\pm$ 15	160 $\pm$ 18 <sup>a</sup>	60 $\pm$ 13 <sup>a</sup>
VLDL	CHOL: $\mu$ g:PL (1:7.4:1)	39.5 $\pm$ 18.7	110 $\pm$ 11	155 $\pm$ 21 <sup>a</sup>	61 $\pm$ 19 <sup>a</sup>
Emul A	$\mu$ g:PL (2.7:1)	35.8 $\pm$ 14.9	95 $\pm$ 14	150 $\pm$ 12 <sup>a</sup>	75 $\pm$ 12 <sup>a</sup>

To obtain the results depicted in Table 3, cells (clone #1 for apoE3-expressing and clone #4 for apoE4-expressing) were incubated for 96 hr in N2 medium alone or containing the indicated concentrations of particles:  $\beta$ -VLDL, 40  $\mu$ g cholesterol/ml medium (this corresponds to 5  $\mu$ g triglyceride/ml medium); VLDL, 5  $\mu$ g triglyceride/ml medium; emulsion A, 5  $\mu$ g triglyceride/ml medium. CHOL = cholesterol;  $\mu$ g = triglyceride; PL = phospholipid. Each data point was obtained by the measurement of 30,40 cells expressing neurites in three separate experiments. The data are the mean  $\pm$  S.E.M. <sup>a</sup> $p$  < 0.010 versus control\*\*\*.

As shown in Table 3, when the Neuro-2a cells secreting apoE3 were incubated with VLDL, they showed an increase in neurite extension, whereas the apoE4-secreting cells in the presence of VLDL showed an inhibition of neurite extension. In other experiments, human LDL and canine apoE HDL<sub>C</sub>, an apoE-enriched plasma high density lipoprotein (HDL) induced by cholesterol feeding and resembling apoE-containing lipoproteins in the CSF (Pitas et al. (1987)), also were used. The apoE3- and apoE4-secreting Neuro-2a cells did not respond to LDL (40  $\mu$ g cholesterol/ml) (i.e., there was no difference in neurite extension as compared with control cells grown in N2 medium

alone). On the other hand, incubation of apoE HDL<sub>C</sub> (40 µg cholesterol/ml) with the apoE4-secreting or apoE3-secreting cells resulted in only a small reduction or increase in neurite extension, respectively (control cells in N2 medium, 100%; apoE4-secreting cells plus HDL<sub>C</sub>, 85,90% of the value obtained with N2 medium; apoE3-secreting cells plus HDL<sub>C</sub>, 110% of the value obtained with N2 medium).

Liposomes and lipid emulsions also were used in an attempt to define the type of lipid vehicle required for the delivery of the apoE. The DMPC emulsion alone or DMPC complexed with cholesterol were incubated with the apoE3- and apoE4-secreting cells for 96 hr at increasing phospholipid concentrations of up to 45 µg phospholipid and 5 µg cholesterol/ml medium (higher concentrations were toxic to the cells).

In these studies, there was no effect on neurite outgrowth with either of the apoE-transfected Neuro-2a cells. Previously, it was shown that apoE complexes with DMPC and mediates high-affinity binding to the LDL receptor. Pitas et al. (1980) J. Biol. Chem. 255:5454-5460. On the other hand, a lipid emulsion particle (emulsion A in Table 3), which was a triglyceride- and phospholipid-containing spherical particle (approximately 35.8 nm), caused a significant enhancement of neurite extension in the apoE3-secreting cells and was associated with an inhibition of outgrowth in the apoE4-secreting cells. Thus, specific combinations of lipids and/or a unique particle size may be required to elicit the apoE isoform, specific effects on neurite outgrowth. It is interesting to note that the delivery of cholesterol to the cells does not appear to be required for the differential effect.

Additional studies using the lipoproteins from bovine CSF suggest that natural lipoproteins in the CNS may mediate the isoform-specific effects of apoE3 and apoE4. As shown in Fig. 5, addition of lipoproteins isolated from CSF ( $d < 1.21$  g/ml) to the cells caused an inhibition of neurite outgrowth from the apoE4-expressing cells and an increase in outgrowth from the apoE3-expressing cells. When CSF lipoproteins were used at a

concentration of 40  $\mu$ g lipoprotein cholesterol/ml, the effect was similar to that obtained using  $\beta$ -VLDL at the same concentration.

5 CSF lipoproteins ( $d < 1.21$  g/ml) were analyzed for protein and cholesterol content and apolipoprotein composition. The ratio of cholesterol to protein was approximately 1:1, similar to data reported for canine CSF. Pitas et al. (1987). The bovine CSF lipoproteins ( $d < 1.21$  g/ml) contained only apoE and apoA-I when separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and visualized by Coomassie Brilliant Blue staining. These results are similar to those reported previously for human and  
10 canine CSF lipoproteins. Pitas et al. (1987); and Roheim et al. (1979) Proc. Natl. Acad. Sci. USA 76:4646-4649.

The ability of the neuroblastoma cells to bind, internalize, and degrade  $\beta$ -VLDL was examined to determine whether the differences in neurite outgrowth in the apoE3- and apoE4-expressing cells was due to a different ability of the secreted apoE3 and apoE4  
15 to stimulate the delivery of apoE and/or lipoprotein lipids to the cells. In these studies,  $^{125}$ I- $\beta$ -VLDL were used to quantitate the binding, uptake, and degradation of the lipoproteins in the Neuro-2a cells. The results are presented in Table 4.

Table 4

20 *Cell association and degradation of  $^{125}$ I- $\beta$ -VLDL by stably transfected and control cells*

Cell type	$^{125}$ I- $\beta$ -VLDL	
	Cell association (ng of lipoprotein protein/mg of cell protein)	Degradation
Control cells	750 $\pm$ 16	2,467 $\pm$ 331
ApoE3-expressing cells	671 $\pm$ 40 <sup>a</sup>	1,945 $\pm$ 219
25 ApoE4-expressing cells	662 $\pm$ 50 <sup>a</sup>	1,788 $\pm$ 188 <sup>b</sup>

To obtain the results depicted in Table 4, cells were incubated for 24 hr in N2 medium alone. The  $^{125}$ I- $\beta$ -VLDL (3  $\mu$ g protein/ml medium) were then added, and after 16 hr at 37°C the lipoprotein cell association (bound and internalized) and degradation by

Neuro-2a cells were measured. The data reported are the mean of two separate experiments performed in duplicate ( $\pm$  S.D.). Control = cells transfected with pSV2*neo* alone. In Table 4, a represents  $<0.05$  versus control and b represents  $<0.01$  versus control.

The results presented in Table 4 indicate that the total amount of cell-associated (bound and internalized)  $^{125}\text{I}$ - $\beta$ -VLDL was very similar in the apoE3- and apoE4-secreting cells (both were slightly lower than that seen in the non-apoE-transfected control cells). The degradation of  $^{125}\text{I}$ - $\beta$ -VLDL by the apoE3- and apoE4-secreting cells was similar. There was a small (but statistically significant) decrease in the degradation of  $^{125}\text{I}$ - $\beta$ -VLDL by the apoE4-secreting cells when compared with the non-apoE-transfected control Neuro-2a cells.

In a parallel experiment, the cells were incubated with DiI-labeled  $\beta$ -VLDL to visualize the internalization of the lipoproteins in the apoE3- and apoE4-secreting cells by fluorescence microscopy. Following internalization, DiI is trapped in the lysosomes, and the fluorescent intensity of the cells, therefore, is proportional to the total amount of lipoprotein internalized and degraded. Pitas et al. (1983). In these studies, no difference in the uptake of DiI-labeled  $\beta$ -VLDL was observed in the apoE3- and apoE4-secreting cells (Fig. 6). Extraction and quantitation of the DiI from cells incubated with DiI-labeled  $\beta$ -VLDL (40  $\mu\text{g}$  of cholesterol per ml) for 16 hr at  $37^\circ\text{C}$  confirmed the visual impression that the uptake of DiI-labeled  $\beta$ -VLDL was similar in the apoE3- and apoE4-secreting cells. The control cells incorporated  $8.9 \pm 0.4$  ng of DiI per mg of cell protein, while the apoE3- and apoE4-expressing cells incorporated  $10.2 \pm 1.0$  and  $10.8 \pm 0.3$  ng of DiI per mg of cell protein, respectively.

To demonstrate that apoE binds to the lipid particles when it is present at the concentrations secreted by the cells, radiolabeled apoE3 or apoE4 was incubated with the  $\beta$ -VLDL, VLDL, or emulsion A for 1 hr at  $37^\circ\text{C}$  (100 ng of apoE with 40  $\mu\text{g}$  of  $\beta$ -VLDL cholesterol or 100 ng of apoE with either 5  $\mu\text{g}$  of VLDL or emulsion A triglyceride) and fractionated by FPLC. Approximately 70% of the apoE was associated with the  $\beta$ -VLDL

and 50% with the VLDL and emulsion A. There was no difference in the amount of apoE3 or apoE4 associated with the lipid particles.

5

## EXAMPLE 2

### Specific Inhibition of apoE Binding to apoE Binding Receptor

To determine which receptor was involved in mediating the differential effects of apoE3 and apoE4 on neurite outgrowth, inhibitors that block the binding and internalization of apoE-enriched lipoproteins by the HSPG-LRP pathway, but not by the LDL receptor pathway, were used. The effect on neurite outgrowth was then determined. Prior to the addition of  $\beta$ -VLDL, the cells were preincubated for 1 hr with either heparinase (20 units/ml) and chlorate (20 mM), with the RAP (5  $\mu$ g/ml), or with lactoferrin (10  $\mu$ g/ml). The binding of apoE-enriched lipoproteins to the LRP requires their initial binding to cell-surface HSPG. Heparinase and chlorate cleave and reduce the sulfation of cell-surface HSPG, respectively. Ji et al. (1993) J. Biol. Chem. 268:10160-10167; and Humphries et al. (1989) Met. Enzymol. 179:428-434. Lactoferrin blocks binding of lipoproteins to both HSPG and LRP, whereas the RAP primarily blocks the binding of apoE-enriched lipoproteins to the LRP. All of these reagents previously have been shown to inhibit the uptake of apoE-enriched  $\beta$ -VLDL by the LRP. Mahley et al. (1994) Ann. N.Y. Acad. Sci. 737:39-52; Ji et al. (1993); Ji et al. (1994a); and Willnow et al. (1992) J. Biol. Chem. 267:26172-26180. As previously shown in Fig. 3,  $\beta$ -VLDL alone stimulated the outgrowth of neurites. The stimulation of neurite outgrowth by  $\beta$ -VLDL was further enhanced in the apoE3-expressing cells and markedly inhibited in the apoE4-secreting cells (Table 5).

25

Table 5

*Effect of chlorate, heparinase, the RAP, and lactoferrin in the presence of  $\beta$ -VLDL on neurite extension from cells stably transfected with apoE3 or apoE4 cDNA*

5	Treatment	Control	ApoE3-expressing	ApoE4-expressing
		% of value obtained with control cells in N2 medium alone		
	N2 alone	100 ± 8	105 ± 10	103 ± 9
	β-VLDL (40 µg cholesterol/ml)	160 ± 13	209 ± 13 <sup>a</sup>	70 ± 4 <sup>b</sup>
10	β-VLDL + chlorate (20 mM) and heparinase (20 units/ml)	159 ± 14	163 ± 20 <sup>c</sup>	138 ± 12
	β-VLDL + RAP (5 µg/ml) <sup>d</sup>	176 ± 11	179 ± 15	160 ± 16
	β-VLDL + lactoferrin (10 µg/ml)	128 ± 16	154 ± 19 <sup>c</sup>	130 ± 12

To obtain the results depicted in Table 5, cells were incubated for 1 hr in N2  
 15 medium alone or containing the indicated concentrations of chlorate, heparinase, RAP, or  
 lactoferrin. Then the  $\beta$ -VLDL were added, and the incubation was continued for a total of  
 96 hr. The reagents, except for  $\beta$ -VLDL, were re-added every 24 hr. The media and  $\beta$ -  
 VLDL were changed at 48 hr. Each data point was obtained by measuring 30,40 neurons  
 expressing neurites in two separate experiments. Data are the mean  $\pm$  S.E.M. <sup>a</sup> $p$  < 0.05,  
 20 <sup>b</sup> $p$  < 0.01 versus value obtained with control cells (non-apoE-expressing cells incubated  
 with  $\beta$ -VLDL). <sup>c</sup> $p$  < 0.05 versus apoE3-expressing cells with  $\beta$ -VLDL alone. <sup>d</sup>In a  
 parallel set of experiments, 5  $\mu$ g/ml of RAP did not block the binding of DiI-labeled LDL  
 to the Neuro-2a cells.

The results depicted in Table 5 indicate that the addition of chlorate and  
 25 heparinase or the RAP did not block the stimulatory effect of  $\beta$ -VLDL on neurite  
 outgrowth in the control cells (Neuro-2a cells not expressing apoE), suggesting that the  
 effect of  $\beta$ -VLDL alone is mediated by the LDL receptor; however, these reagents  
 blocked the isoform-specific effects in the cells secreting apoE (Table 5). Chlorate and  
 heparinase treatment of the cells or the addition of the RAP prevented the stimulation of

neurite extension in the apoE3-expressing cells incubated with  $\beta$ -VLDL (that is, significantly decreased the  $\beta$ -VLDL, induced neurite extension in the Neuro-2a cells secreting apoE3). Moreover, chlorate and heparinase or the RAP blocked the inhibition of neurite extension seen in the apoE4-expressing cells (that is, the apoE4-expressing cells in the presence of  $\beta$ -VLDL did not demonstrate inhibition of neurite extension but, in fact, showed increased extension) (Table 5). In the presence of heparinase and chlorate or the RAP, in the apoE-secreting cells, neurite outgrowth was similar to that observed when  $\beta$ -VLDL were added to the control cells in the absence of apoE (Table 5). Therefore, in the presence of these reagents, the LDL receptor, mediated effect of  $\beta$ -VLDL was not blocked. Lactoferrin also blocked the effects of apoE3 and apoE4 on neurite outgrowth; however, it also slightly suppressed the effect of  $\beta$ -VLDL on neurite extension in the control cells. These data show that inhibition of the interaction between  $\beta$ -VLDL and the HSPG-LRP pathway prevents the differential effects of apoE3 and apoE4 on neurite outgrowth (Table 5).

In dorsal root ganglion or neuroblastoma cells, apoE3 plus a source of lipid supports and facilitates neurite extension. ApoE3 appears to accumulate widely in cell bodies and neurites, stabilize the cytoskeleton and support neurite elongation, and directly or indirectly modulate microtubule assembly. ApoE4, on the other hand, does not appear to accumulate within neurons or support neurite extension, and may even destabilize the microtubule apparatus. The apoE4 effect appears to be mediated via the LRP pathway. Individuals with apoE4 clearly have normal neuronal development early in life. However, apoE4 may exert its detrimental effects later in life, by not allowing or supporting remodeling of synaptic connections. This effect may be important in the pathogenesis of Alzheimer's disease. Alternatively, apoE4 may contribute to Alzheimer's disease by aiding the formation of dense, complicated, possibly toxic plaques of A $\beta$  peptide. At present, the pathway whereby apoE affects the development of Alzheimer's disease remains speculative.

## EXAMPLE 3

Methods of detection of agents that interfere with the apoE4 domain interaction

5 ApoE4 is iodinated using the Bolton-Hunter reagent (New England Nuclear Corp., Boston, MA) as previously described by Innerarity et al. (1979) J. Biol. Chem. 254:4186-4190, with specific activities ranging from 200 to 1100 dpm/ng. The iodinated apoE4 (0.5-2 mg in 50-10 ml 0.1 M  $\text{NH}_4\text{HCO}_3$ ) is incubated with the test reagent or compound and the mixture is added to 250 ml of plasma from normal subjects at 37°C for 2 h.

10 Plasma is then fractionated into the various lipoprotein classes by chromatography on a Superose 6 column (10/50 HR, Pharmacia Fine Chemicals, Uppsala, Sweden) eluted with 20 mM sodium phosphate (pH 7.4), containing 0.15 M NaCl. The column flow rate is 0.5 ml/min, 0.5 ml fractions are collected, and the  $^{125}\text{I}$  content is determined in a Beckman 8000 gamma counter (Beckman Instruments, Fullerton, CA). Reagents that interfere with

15 apoE4 domain interaction will shift the preference of the "modified" apoE4 from VLDL to HDLs, resulting in a distribution that resembles that of apoE3 (run in parallel as a control).

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications may be practiced.

20 Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

25

## WHAT IS CLAIMED IS:

- 5 1. A method of reducing apolipoprotein E4 (apoE4)-induced inhibition of neuron remodeling comprising administering to a patient in need thereof an effective amount of a therapeutic agent wherein the therapeutic agent prevents apoE4 from interacting effectively with neuronal low density lipoprotein receptor-related protein or related apoE binding receptors.
- 10 2. The method according to claim 1, wherein the patient is suffering from a disorder selected from the group consisting of neurodegeneration and hypoxia.
3. The method according to claim 2, wherein the neurodegeneration is due to Alzheimer's disease.
4. The method according to claim 2, wherein the neurodegeneration is due to trauma.
- 15 5. The method according to claim 2, wherein the hypoxia is temporary.
6. The method according to claim 2, wherein the hypoxia is due to stroke.
7. The method according to claim 2, wherein the neurodegeneration is caused by viral infections, genetic enzyme deficiencies, age-related cognitive decline, and prior diseases.
- 20 8. The method according to claim 7, wherein the viral infection is due to a virus selected from the group consisting of human immunodeficiency virus and Epstein-Barr virus.
9. The method according to claim 7, wherein the genetic enzyme deficiencies is a deficiency in  $\beta$ -N-acetylhexosaminidase.
- 25 10. The method according to claim 7, wherein the prion diseases are selected from the group consisting of Kuru and Creutzfeldt-Jacob disease.
11. The method according to claim 1 wherein apoE4 is prevented from interacting effectively by prevention of binding to with neuronal low density lipoprotein receptor-related protein or related apoE binding receptors.

12. The method according to claim 11, wherein the therapeutic agent is selected from the group consisting of receptor-associated protein, analogs and functional fragments thereof, monoclonal antibodies and effective fragments thereof, modified  
5 heparan sulfate proteoglycan and lactoferrin and effective analogs and fragments thereof.

13. The method according to claim 1 wherein apoE4 is prevented from interacting effectively by altering the apoE4 domain interaction to interfere with inhibition of apoE4 in neuron remodeling.

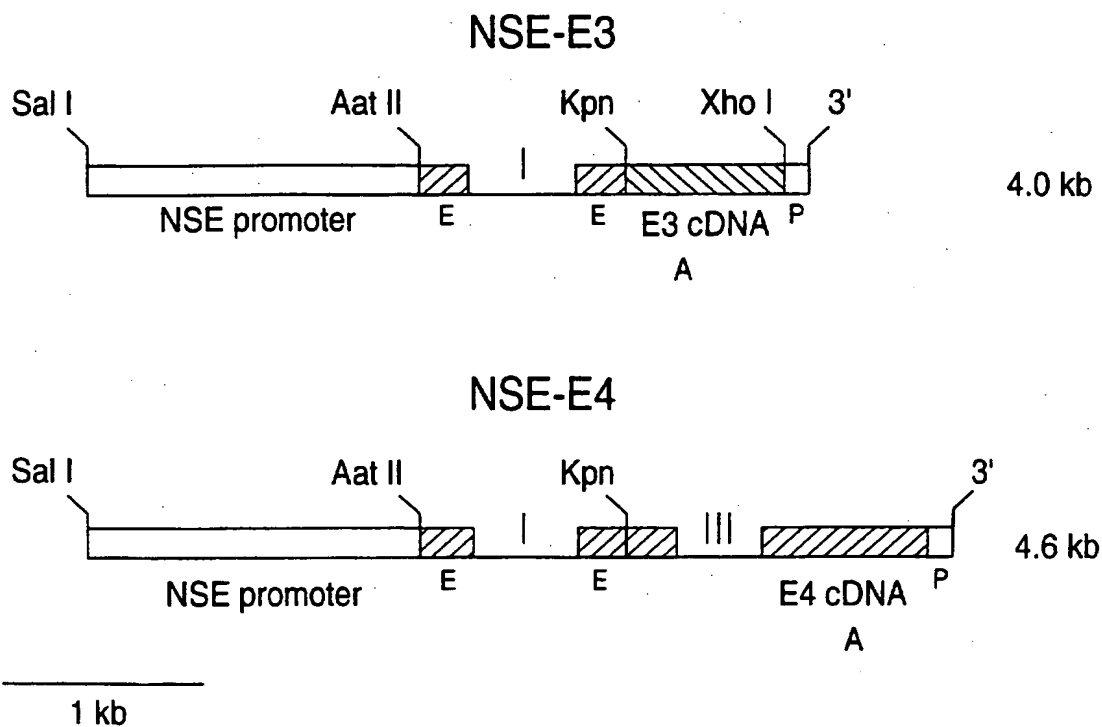
10 14. The method according to claim 13 wherein the therapeutic agent is selected from the group consisting of small molecules, peptides and antibodies which bind to arginine-61 or glutamic acid-255 of apoE4.

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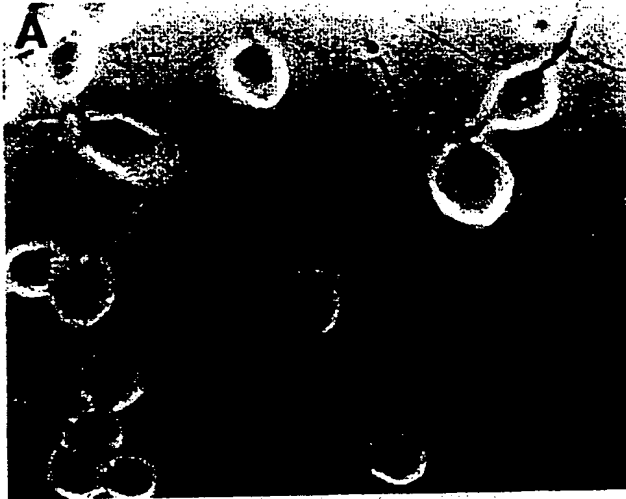
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**FIG. 1**

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**FIG. 2A**

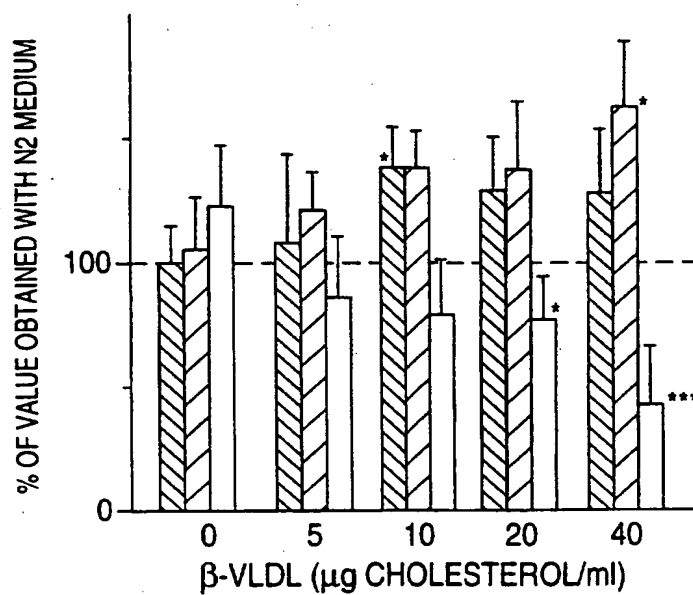
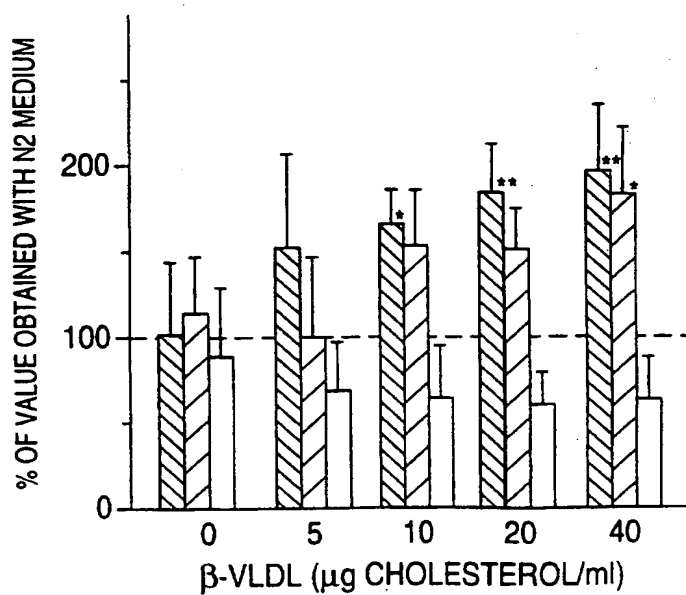


**FIG. 2B**



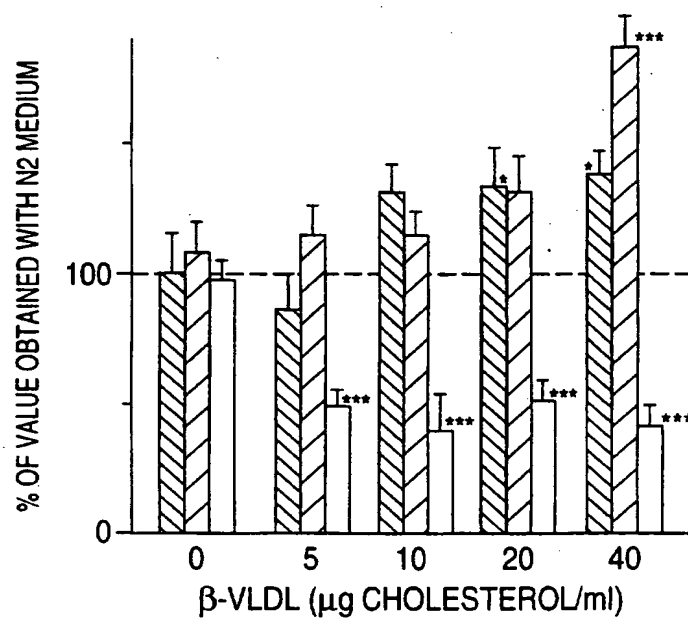
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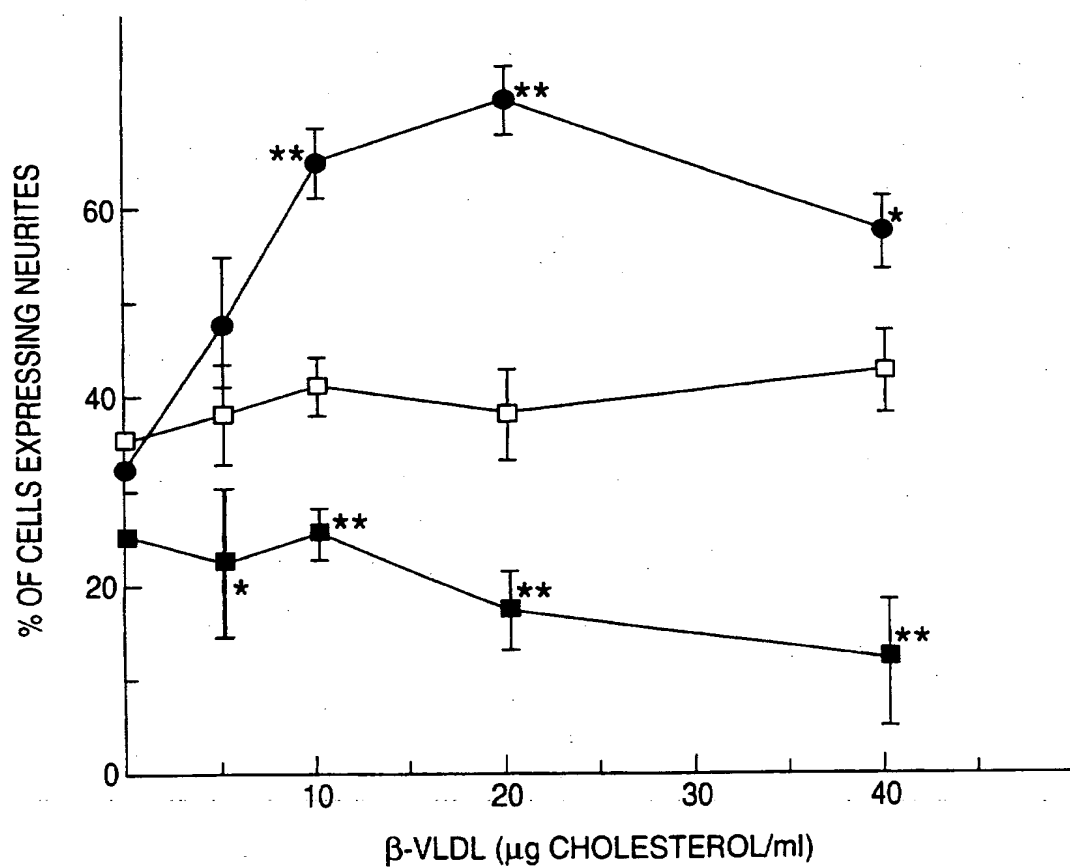
**FIG. 3A****FIG. 3B**

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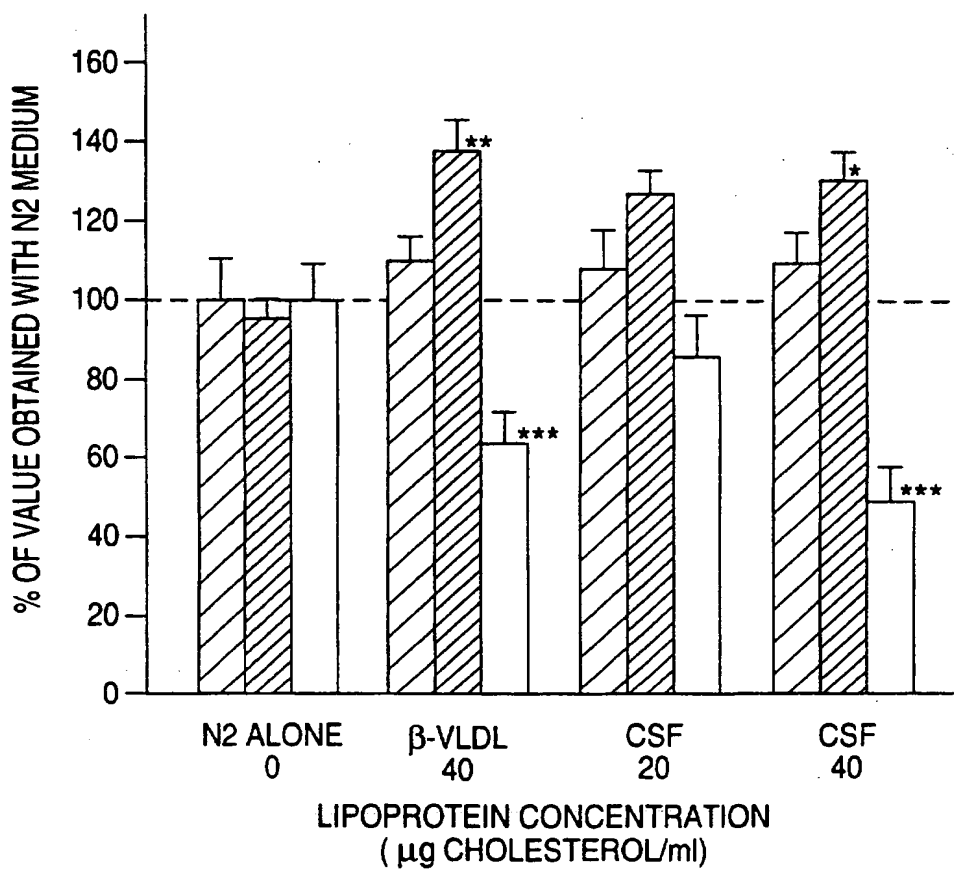
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**FIG. 3C**

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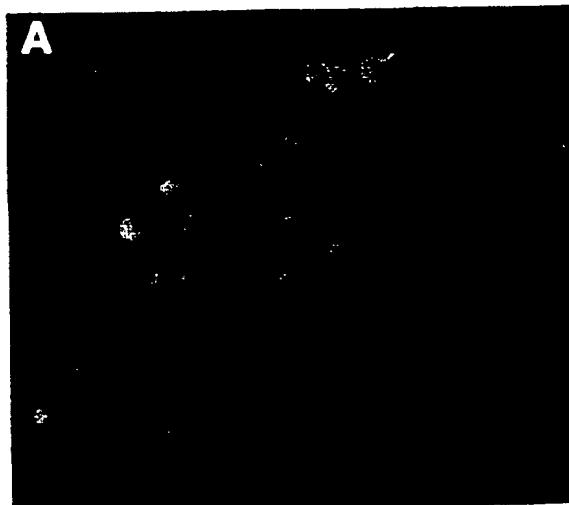
**FIG. 4**

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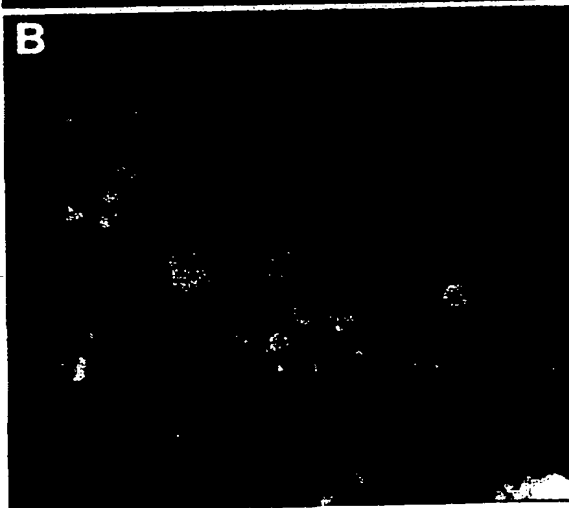
**FIG. 5**

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**FIG. 6A**



**FIG. 6B**



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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/02447

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC(6) : A61K 39/395, 39/40, 39/42, 38/00, 38/16, 31/725 US CL : 424/130.1, 139.1, 143.1, 156.1; 514/2, 8, 56 According to International Patent Classification (IPC) or to both national classification and IPC				
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) U.S. : 424/130.1, 139.1, 143.1, 156.1; 514/2, 8, 56 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Please See Extra Sheet.				
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>				
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.		
Y	Ann. Neurol, Volume 37, Number 2, issued February 1995, Rebeck et al, "Multiple, Diverse Senile Plaque-associated Proteins Are Ligands of an Apolipoprotein E Receptor, the $\alpha$ 2-Macroglobulin Receptor/Low-Density-Lipoprotein Receptor-related Protein", pages 211-217, see entire document.	1-14		
Y	Society for Neuroscience, Volume 21, issued 1995, Ma et al, "Promotion Of The Neurotoxicity Of Alzheimer AB Protein By The Pathological Chaperones ACT And APOE4: Inhibition By AB-Related Peptides And APOE2", page 1714, abstract 670.7, see entire document.	1-14		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.				
<table border="0"> <tr> <td>           * Special categories of cited documents:            "A" document defining the general state of the art which is not considered to be of particular relevance            "E" earlier document published on or after the international filing date            "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)            "O" document referring to an oral disclosure, use, exhibition or other means            "P" document published prior to the international filing date but later than the priority date claimed         </td> <td>           "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention            "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone            "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art            "&amp;" document member of the same patent family         </td> </tr> </table>			* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family
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Date of the actual completion of the international search 17 JUNE 1996		Date of mailing of the international search report 12 JUL 1996		
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer <i>Deborah Duffy</i> PATRICIA A. DUFFY Telephone No. (703) 308-0196		

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/02447

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Volume 264, issued 06 May 1994, Nathan et al, "Differential Effects of Apolipoproteins E3 and E4 on Neuronal Growth in Vitro", pages 850-852, see entire document.	1-3, 7, and 11-14
Y	Society For Neuroscience, Volume 21, issued 1995, Beffert et al, "Apolipoprotein E Uptake Is Increased In The Presence Of Beta Amyloid Peptides And Reduced By Blockade Of The Low Density Lipoprotein Receptor", page 6, abstract 9.6, see entire abstract.	1-3 and 12-13
Y	Lancet, Volume 346, Number 8974, issued 26 August 1995, Alberts et al, "ApoE genotype and survival from intracerebral haemorrhage", page 575, see entire document.	4-6
Y	The Journal of Biological Chemistry, Volume 269, Number 4, issued 28 January 1994, Ji et al, "Secretion-Capture Role for Apolipoprotein E in Remnant Lipoprotein Metabolism Involving Cell Surface Heparan Sulfate Proteoglycans", pages 2764-2772, see entire document.	12 and 14
A	FEBS Letters, Volume 338, issued 1994, Lopes et al, "Expression of $\alpha$ 2-macroglobulin receptor/low density lipoprotein receptor-related protein is increased in reactive and neoplastic glial cells", pages 301-305, see entire document.	1-14
Y --- A	Current Opinion in Structural Biology, Volume 4, issued 1994, Weisgraber et al, "Lipoproteins, neurobiology, and Alzheimer's disease: structure and function of apolipoprotein E", pages 507-515, see entire document.	1-3 ----- 4-14
Y	Proc. Natl. Acad. Sci., Volume 91, issued April 1994, Orlando et al, "Functional domains of the receptor-associated protein (RAP)", pages 3161-3165, see entire document.	12
A	The Journal of Biological Chemistry, Volume, 269, Number 35, issued 02 September 1994, Dong et al, "Human Apolipoprotein E Role Of Arginine 61 In Mediating The Lipoprotein Preferences Of The E3 And E4 Isoforms", pages 22358-22365, see entire document.	14

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/02447

### B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DIALOG, MEDLINE, EMBASE, DERWENT WPI, CAB ABSTRACTS, JAPIO

search terms: inventors, neuron, neural, neuronal, apoE, apolipoprotein E, therapy or therapeutic, degeneration, inhibition, remodeling, receptor, antibody, immunoglobulin, receptor-associated protein, heparin sulfate, lactoferrin, conformation, activity, arginine-61 or glutamic acid-255, Alzheimer's, trauma, stroke, neurodegenerative disease.